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Paternity analysis of wild-caught females shows that sperm package size and placement influence fertilization success in the bushcricket *Pholidoptera griseoaptera*

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Abstract

In species where females store sperm, males may try to influence paternity by the strategic placement of sperm within the female's sperm storage organ. Sperm may be mixed or layered in storage organs, and this can influence sperm use beyond a 'fair raffle'. In some insects, sperm from different matings is packaged into discrete packets (spermatodoses), which retain their integrity in the female's sperm storage organ (spermatheca), but little is known about how these may influence patterns of sperm use under natural mating conditions in wild populations. We examined the effect of the size and position of spermatodoses within the spermatheca and number of competing ejaculates on sperm use in female dark bushcrickets (*Pholidoptera griseoaptera*) that had mated under unmanipulated field conditions. Females were collected near the end of the mating season, and seven hypervariable microsatellite loci were used to assign paternity of eggs laid in the laboratory. Females contained a median of three spermatodoses (range 1–6), and only six of the 36 females contained more than one spermatodose of the same genotype. Both the size and relative placement of the spermatodoses within the spermatheca had a significant effect on paternity, with a bias against smaller spermatodoses and those further from the single entrance/exit of the spermatheca. A higher number of competing males reduced the chances of siring offspring for each male. Hence, both spermatodose size and relative placement in the spermatheca influence paternity success.

Keywords: cryptic female choice, polyandry, postcopulatory sexual selection, sperm competition, spermatodose

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Introduction

Polyandry (females mating with more than one male) is taxonomically widespread (Simmons 2005; Taylor *et al.* 2014) and can result in intense postcopulatory sexual selection, in the form of both sperm competition and cryptic female choice (Eberhard 1996; Birkhead & Møller 1998; Simmons 2001, 2014; Arnqvist 2014). Sperm competition (competition between the sperm of two or more males for the fertilization of the female's eggs) has resulted in numerous male adaptations to maximize paternity, including traits that allow a male to displace or remove rival sperm from the female's reproductive tract and to deter the female from mating with other males (Birkhead & Møller 1998; Simmons 2001, 2014).

The outcome of postcopulatory sexual selection, in terms of which male's sperm is used to fertilize the majority of a multiply-mated female's eggs, has usually been studied by mating females with two different

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males in a laboratory setting and is often expressed as the proportion of offspring sired by the last male to mate, or P_2 (Birkhead & Møller 1998; Simmons 2001). Laboratory-based studies have identified a wide range of factors that can determine variation in patterns of sperm use (Birkhead & Møller 1998; Simmons 2001, 2014; Droge-Young *et al.* 2016). Mating order is one such factor. In the majority of insect species, for example, the last male to mate with the female tends to fertilize the greater proportion of her eggs (i.e. there is last-male sperm precedence) (Simmons & Siva-Jothy 1998; Simmons 2001, 2014), although patterns of sperm precedence can vary widely, even between closely related species. In the bushcrickets or katydids (Orthoptera: Tettigoniidae), for example, reported patterns of sperm precedence in the laboratory range from first-male priority (Simmons & Achmann 2000), sperm mixing (Wedell 1991) to pronounced last-male sperm precedence (von Helversen & von Helversen 1991; Achmann *et al.* 1992; Vahed 1998). In some cases, mating order can affect the outcome of sperm precedence due to its effect on the relative positioning of sperm from different males in the female's reproductive tract (Simmons & Siva-Jothy 1998; Droge-Young *et al.* 2016). It has been suggested that in insects, sperm from different males may sometimes become stratified within the female's sperm stores as a result of their elongated shape, leading to a 'last in, first out' mechanism of sperm precedence (Simmons & Siva-Jothy 1998). In a few species, such as the dragonfly *Crocothemis erythraea* (Odonata: Libellulidae), males can influence the process of stratification using inflatable structures on their intromittent organ to push rival sperm to the back of the sperm storage organ prior to transferring their own sperm (Siva-Jothy 1988). Due to the difficulty of distinguishing sperm from different males within the female's sperm stores, however, very few previous studies have been able to quantify the effect of the relative position of sperm on male fertilization success (e.g. see Manier *et al.* 2010, 2013a,b; Droge-Young *et al.* 2016).

In many animals, individual sperm do not mix freely within the reproductive tract of the female, but instead occur in discrete aggregations or bundles (spermatodesmata) or in capsules that enclose the sperm from individual males within the female's sperm storage organ (spermatodoses, not to be confused with spermatophores, the packages males use to transfer sperm to the female) (Mann 1984; Higginson & Pitnick 2011; Fisher *et al.* 2014). Spermatodoses, or spermatodose-like structures, occur in numerous insect families in several orders including Orthoptera, Phthiraptera, Psocoptera, Thysanoptera and Hemiptera (Vahed 2003; Marchini *et al.* 2012). In bushcrickets, spermatodoses are thought to form within the female's spermatheca (sperm storage organ) from secretions that are transferred from the externally attached spermatophore before the sperm mass (Vahed 2003). Because one spermatodose appears to be formed per mating and spermatodoses remain intact throughout the female's adult life, spermatodose counts have been used to estimate the degree of polyandry in field-mated bushcrickets (Gwynne 1984; Vahed 2006; Robson & Gwynne 2010; Vahed *et al.* 2011; Kaňuch *et al.* 2013; Jarčuška & Kaňuch 2014). However, their influence on paternity has not been studied. In bushcrickets, each spermatodose has a spherical body with a double-layered outer wall surrounding a tightly coiled ball of sperm, arranged in feather-like spermatodesmata. Emerging from the body of the spermatodose is an elongated, tubular exit (Viscuso *et al.* 2002; Vahed 2003). In certain bushcricket species, such as *Pholidoptera griseoaptera*, the spermatodoses from different matings become stratified within the elongated spermatheca of the female (Vahed 2003; Fig 1). It has been proposed that spermatodoses and other aggregations of sperm could function to block the exit of rival sperm from the spermatheca, while allowing the male to deploy his sperm strategically in a position closest to the exit of the spermatheca (Simmons & Siva-Jothy 1998; Vahed 2003); however, this hypothesis has not been tested. This hypothesis predicts that a high level of last-male sperm precedence should occur in spermatodose-producing species.

A further factor that can affect patterns of sperm use is relative ejaculate size (Simmons 2001, 2014). Laboratory studies of a range of taxa have found that when a female has mated with two different males, the relative amount of sperm received from a given male determines the proportion of eggs that he subsequently fertilizes (Martin *et al.* 1974; Simmons 1987; Parker *et al.* 1990; Gage & Morrow 2003; but see also Snook 2005). We are not aware of any previous studies that have examined the effect of natural variation in ejaculate size on patterns of sperm use in field-mated females.

Laboratory studies of factors associated with sperm precedence are unlikely to reflect conditions experienced in the field, such as the females' natural number of mates and natural remating intervals (Zeh & Zeh 1994; Simmons 2001; Lewis *et al.* 2005; Oneal & Knowles 2015). Zeh & Zeh (1994), for example, found that, in a species of pseudoscorpion (*Cordylochernes scorpioides*), last-male sperm precedence broke down when females were mated with more than two males. The nature of the social group within which *Drosophila melanogaster* occur can also influence both the remating rate and paternity success of males in surprisingly complex ways (Billeter *et al.* 2012). The degree of polyandry and paternity skew (i.e. inequality among paternity shares) has been quantified in females that have mated with

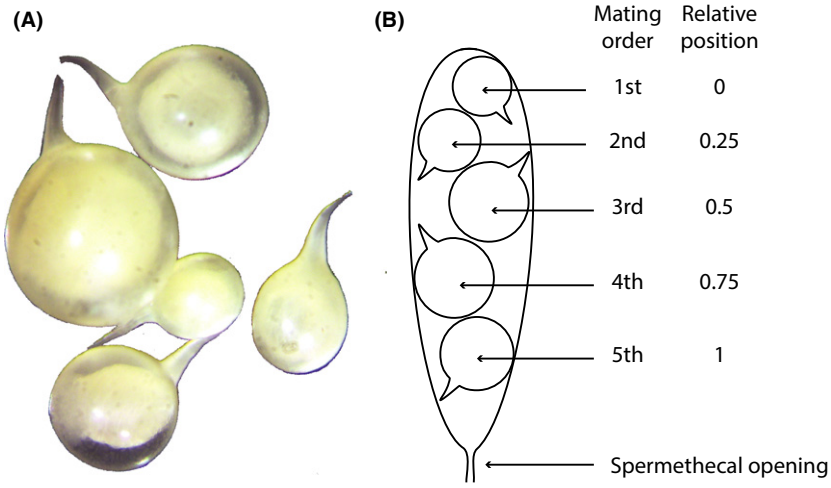

Fig. 1 (A) Photograph of dissected spermatodoses from *P. griseoaptera* (mean diameter = 0.90 mm). (B) Schematic diagram of a longitudinal section through the spermatheca in *P. griseoaptera*, showing how the relative position of each spermatodose within the spermatheca was scored.

multiple males under natural field conditions using hypervariable molecular markers (Taylor *et al.* 2014) in a variety of taxa including, for example, arthropods such as crickets and bushcrickets (Orthoptera: Ensifera; Bretman & Tregenza 2005; Hockham *et al.* 2004; Simmons *et al.* 2007; Simmons & Beveridge 2010; Turnell & Shaw 2015a,b; Oneal & Knowles 2015). Some studies of vertebrates, such as those of feral Soay sheep, *Ovis aries* (Preston *et al.* 2003), have additionally used direct observations of mating in the field to examine factors that affect patterns of sperm use in field-mated females. In many arthropod species, however, such field observations are often not practical due to their small size, high mobility and/or cryptic nature. Consequently, very few previous studies of arthropods (e.g. see Rodríguez-Munoz *et al.* 2010; Turnell & Shaw 2015b) have been able to examine factors that affect patterns of sperm use in females that have mated with multiple males under natural field conditions.

Here, using a species in which sperm from different matings occur in discreet aggregations (spermatodoses) within the spermatheca (the bushcricket *Pholidoptera griseoaptera*), we were able to examine the influence of the position, size and number of spermatodoses within the female spermatheca on patterns of sperm use in females that had mated under unmanipulated, natural field conditions.

Methods

The study species

The dark bushcricket, *Pholidoptera griseoaptera* (DeGeer, 1773), is common and widespread in Europe, where it is often associated with forest clearings, woodland edges and hedgerows (Benton 2012). The eggs, which are laid in the summer and autumn, hatch in either the spring of the following year or the one after (Hartley & Warne 1972; Benton 2012). After passing through six to seven nymphal instars, individuals become adult in mid- to late July (Benton 2012; Kaňuch *et al.* 2015). The peak of mating activity occurs in August (Kaňuch *et al.* 2015), but individuals can survive into the late autumn (Benton 2012). Both sexes are flightless, but nevertheless have good dispersal ability (Diekötter *et al.* 2005).

Males attract females by tegminal stridulation and both sexes mate multiple times (Benton 2012; Kaňuch *et al.* 2015). In common with most other bushcrickets, the male transfers a large externally visible spermatophore to the female towards the end of copulation. The spermatophore represents approximately 11% of male body mass in this species and consists of two parts: the ampulla which contains the ejaculate and the gelatinous spermatophylax which the female consumes during ejaculate transfer (Vahed *et al.* 2014). As in other bushcrickets, both the male and female enter a nonreceptive sexual refractory period following each mating (Vahed 2007). The mean (±SE) sexual refractory period for females is 117.57 ± 15.62 h, while that for the males is 27.67 ± 6.94 h (see Supporting information).

Population sampling

A total of 38 female *P. griseoaptera* were collected from a field site near Silverton, Devon, UK, towards the end of the mating season from 5 to 12 September 2009. The field site consisted of a 50-m-long stretch of roadside verge and hedge bank (grid reference SS 95540 00570), at an altitude of approximately 43 m above sea level. Females were taken back to the laboratory and kept in separate cylindrical cages (17 cm high by 8 cm in diameter). Each cage was provided with food in the form of wheatgerm, together with young dock (*Rumex* sp.) and buttercup (*Ranunculus* sp.) leaves. A block of flower-

arranging 'Oasis' polyurethane foam (Smithers-Oasis, USA), cut to 3 × 8 × 3 cm, was provided as an oviposition medium. Females were allowed to lay eggs for 14 days before being frozen at −80 °C until dissection and DNA extraction. The eggs were extracted by crumbling the foam through a nylon sieve. The mean number of eggs laid per female over the 2-week period was 56 (range: 21–85). Eggs from each female were placed in Petri dishes containing damp cotton wool, covered by a disc of filter paper. Eggs were maintained at 25 °C for 3 months, after which the degree of development of the embryos was scored. In *P. griseoaptera*, eggs can either enter obligate winter diapause at the whole-embryo stage (in which the embryo occupies the whole of the egg and the eyes are clearly visible towards the end of the egg), or as an early embryo (in which little embryonic development is visible) (Hartley & Warne 1972). In our study, approximately 40% of viable eggs, on average, developed to the whole-embryo stage after 3 months of incubation, while the remainder were at the early embryo stage. There were very few unviable eggs in our samples. Twenty whole-embryo eggs were collected at random from each Petri dish (i.e. from each female). Whole embryos were selected simply to maximize the amount of DNA available. If sufficient whole-embryo eggs were not available, eggs with early embryos were substituted. These were stored in 100% ethanol at −80 °C prior to DNA extraction.

Dissection of spermatodoses

After thawing, the spermatheca was dissected from the female and placed in a drop of water in a Petri dish. The spermatheca itself was then dissected by removing the spermathecal wall using mounted needles under a light-dissecting microscope, working upwards from the exit of the spermatheca. Each spermatodose was extracted as it emerged and the diameter of each spermatodose was measured. The walls of the spermatodose are rigid, and the diameter of the spermatodose does not decrease as sperm exit. Consequently, spermatodose diameter is likely to reflect the volume of sperm transferred by that male. The relative position of each spermatodose within the spermatheca in relation to the opening of the spermathecal exit was also recorded. Although spermathecal walls are flexible, the spermatheca of this species is elongated, resulting in the stratification of spermatodoses within the spermatheca (Fig. 1B). This allows us to determine the order in which each spermatodose was deposited (Vahed 2003). For the statistical analysis, the relative position of each spermatodose was recorded as '1' for the one closest to the spermathecal opening (i.e. the last male to mate) and '0' for the one furthest from the spermathecal opening (i.e. the first male to mate). If there were more than two spermatodoses, the spermatodoses in between the two extreme ends of the spermatheca were scored as fractions. For example, for four spermatodoses, the order was recorded as: '0, 0.33, 0.67, 1' while for five spermatodoses, the order was recorded as: '0, 0.25, 0.5, 0.75, 1' (Fig. 1B). Each spermatodose was stored individually in an Eppendorf tube containing 100% ethanol and maintained at −80 °C prior to DNA extraction.

DNA extraction

For the females, we extracted DNA from 10 to 20 mg of hind-leg muscle tissue. For offspring, we used whole embryos. DNA extraction from females and embryos was conducted following standard molecular protocols. To extract DNA from spermatodoses, we used a protocol adapted from Simmons *et al.* (2007), which firstly removes DNA from any female cells that may be present in the sample, before extracting male DNA from the spermatodose (for details, see Supporting Information).

Microsatellite analysis

We used six microsatellite primer pair sequences from Arens *et al.* (2005), chosen on the basis of their reported variability and fragment size. We used 5′ fluorescent-dye labelled/unlabelled primer pairs (Life Technologies) to allow multiplexing of microsatellites (see Table 1). Note the same dye colour was used for WPG10-1 and WPG1-28, and WPG2-30 and WPG8-2 as these can easily be distinguished as they have different size ranges. Also note that primer pair WPG1-27 amplifies two microsatellite loci as described in Arens *et al.* (2005) meaning that samples were genotyped at a total of seven microsatellite loci. Microsatellites were amplified with the Qiagen Multiplex PCR kit following the

Table 1 Properties of the of the six microsatellite markers used in the paternity analysis [for primer sequences, see Arens *et al.* (2005)]

Locus name	Number of alleles	Length (bp)	Dye label
WPG10-1	3	123–129	VIC
WPG1-28	32	267–543	VIC
WPG2-30	3	147–174	PET
WPG8-2	9	217–286	PET
WPG2-15	7	240–258	FAM
WPG1-27 (a)*	3	189–229	NED
WPG1-27 (b)*	14	268–307	NED

*Note primer pair WPG1-27 amplifies two microsatellite loci (Arens *et al.* 2005) (denoted a and b here).

manufacturer's instructions. The amount of primer used for each microsatellite was optimized so that each product showed similar amplification (final ratio used: WPG 10_1: WPG 1_28: WPG 2_30: WPG 8_2: WPG 2_15: WPG 1_27 = 1.00: 1.50: 2.25: 4.50: 1.50: 1.50). Microsatellites were amplified using a G-Storm GS1 thermocycler with the following program: denature at 95 °C for 15 min, followed by 30 cycles at 94 °C for 2 min, 60 °C for 1.5 min, 72 °C for 1 min, followed by a final extension time of 30 min at 60 °C. Extension products were resolved on an ABI 3730XL machine performed by Edinburgh Genomics (https://genomics.ed.ac.uk/). Alleles were sized to an internal size standard (GeneScan-500 LIZ; Applied Biosystems) using Peak Scanner v2.0 (Applied Biosystems) and corrected manually where necessary.

Genotyping failure rate by loci

One spermatodose (from a total of 115) and six offspring (from a total of 693) were unable to be genotyped at any of our microsatellite markers and likely represent DNA extraction failures. For the remaining samples, one was genotyped only at three loci, four at four loci, with the remainder all being genotyped for at least five loci (mean number of loci genotyped per individual = 6.31). The rate of genotyping success was not uniform across loci, with some having a genotype success rate of near 100% while others were below 60% (Table 2). These loci were retained despite their high failure rate as they still provided useful paternity information.

Paternity analysis

Paternity analysis was conducted using r package MasterBayes (version 2.52) in r (R Core Team (2016), version 3.3.0). MasterBayes uses a Bayesian, consistent full-probability model approach that allows paternity information and values of parameters of interest to be estimated simultaneously (Hadfield *et al*. 2006). The genotypes for the seven microsatellite loci, along with phenotypic information for relative mating order, and spermatodose size were provided to MasterBayes to assign paternity to each offspring, and estimate the effect of relative mating order and spermatodose size on the probability of siring offspring. MasterBayes was run using default priors for 1 100 000 iterations with a burn-in of 100 000 iterations, and thinning interval of 10. Dropout and stochastic error rates were fixed at 0.005. Mean values for the parameters of interest (relative mating order and spermatodose size) were estimated from 100 000 MCMC samples from the posterior distribution, which were also used to obtain a 95% credible interval (highest posterior density interval) for these parameters.

To further examine these relationships, we used the offspring for which the posterior probability of the most likely father was >0.9. From this, we calculated the number of offspring each male sired as a proportion of those successfully assigned to any father. In six of the females, two of the spermatodoses in the female's spermatheca had the same genotype, meaning offspring produced from spermatodoses with this genotype could not be assigned to an individual spermatodose. As a result, these spermatodoses were discarded from subsequent analyses. Note that since the number of offspring that were produced from either of these spermatodoses is known, the correct proportion of offspring sired from the other spermatodoses in the spermatheca could be correctly calculated and were thus retained in the GLM analysis (below).

We then calculated paternity skew (sensu Pamilo & Crozier 1996) per female as follows: paternity skew = (total number of males $- 1/(\Sigma x^2)$)/(total number of males $- 1$), where x is proportion of offspring sired by a male. This measure of paternity skew gives a value between 0 and 1 where 1 indicates a completely unequal paternity share (one father sires all the offspring) and a value of 0 indicates shared paternity (i.e. all fathers sire equal numbers of offspring). We then tested whether the observed paternity skew was significantly different than equal paternity (0) using a one-sided, one-sample sign test in r (R Core Team (2016), version 3.3.0). Note, for the calculation of paternity skew, females which had any offspring assigned to a spermatodose with a duplicate genotype in the same spermatheca (see above) were discarded.

We then determined which factors influenced the proportion of offspring sired using a quasi-Poisson general linear model (GLM) in r (R Core Team (2016), version 3.3.0) with the following terms: number of competing males, spermatodose size, and relative

Table 2 Percentage genotyping success for the microsatellite loci used in the paternity analysis. Note primer pair WPG1-27 amplifies 2 microsatellite loci (Arens *et al*. 2005) (denoted a and b here)

Microsatellite	Samples genotyped (*N*)	Samples genotyped (%)
WPG10-1	837	100.0
WPG1-28	835	99.8
WPG2-30	834	99.6
WPG8-2	488	58.6
WPG2-15	836	99.9
WPG1-27 (a)	647	77.5
WPG1-27 (b)	808	96.6

mating order and all their possible interactions. Model simplification was then conducted by dropping the highest least-significant term from the model until a term had a *P*-value of <0.05. Following this, we then examined quadratic terms for number of competing males, relative mating order and spermatodose size by adding these factors into the model one-by-one. If the added quadratic term was significant ($P < 0.05$), it was retained.

Results

Polyandry

All of the 38 females collected in the study were found to have mated (i.e. showed the presence of a spermatodose in the spermatheca) (mean number of spermatodoses = 3.08; median = 3). However, two females were found to have mated only once (Table 3) and thus were excluded from paternity analyses (below). We found no correlation between number of spermatodoses and female size (pronotum length) or fecundity (number of eggs laid) (r_s for pronotum length = 0.011, $P = 0.95$; r_s for number of eggs laid = 0.209, $P = 0.21$). Spermatodose size ranged from 0.50 mm to 1.40 mm in diameter (mean = 0.90 mm) and was not correlated with mating order ($r_s = 0.056$, $P = 0.555$). There was no significant correlation between the number of spermatodoses and either the diameter of the spermatodose nearest to the blind end of the spermatheca ($r_s = -0.163$, $P = 0.33$) or mean spermatodose diameter ($r_s = -0.144$, $P = 0.40$).

Paternity analysis

Both relative mating order and spermatodose size have a significant effect on the likelihood of siring offspring (Table 4). We found that the chance of siring offspring increased with spermatodose size and male mating order (as inferred from relative spermatodose position in the spermatheca), with males mating later in the mating order siring more offspring. To examine these relationships in more depth, we conducted additional analyses on those offspring for which the posterior probability of the most likely father was >0.9, which totalled 496 of the 693 offspring analysed.

Table 3 Number of spermatodoses present in females

Number of spermatodoses	Number of females
0	0
1	2
2	13
3	10
4	8
5	3
6	2

Table 4 Parameter estimates from MasterBayes using a 100 000 MCMC samples from the posterior distribution, showing the effect of relative spermatodose order within the spermatheca and spermatodose diameter on the likelihood of siring offspring (HPD, highest posterior density)

Parameter	Posterior mean (95% HPD)
Relative mating order	0.793 (0.544–1.042)
Spermatodose diameter	8.164 (6.910–9.417)

Overall we found that paternity was highly skewed away from equal paternity (median paternity skew = 0.92). Paternity skew was significantly higher than the value expected for equal paternity (0) (one-sample sign test *P*-value = $3.559\ 10^{-08}$). This pattern was consistently found regardless of the number of competing males (Fig. 2). The observed value of paternity skew was significantly higher than that expected for equal paternity when the numbers of competing males were 2, 3 or 4 (one-sample sign test *P*-values = 0.0004, 0.0038, 0.0368, respectively) but not 5 or 6 (one-sample sign test *P*-values >0.05) likely due to the small number of females in these categories. Taken together, these results show that paternity share is highly skewed towards a small number of males.

To examine the possible causes of this paternity skew, we then used a quasi-Poisson GLM to determine the effect of the number of competing males, spermatodose size and relative mating order on the proportion of offspring sired. Results are summarized in Table 5. Note fitting interactions between number of competing males, spermatodose size and relative mating order were not significant ($P > 0.35$) and so these terms were dropped. We also found that quadratic terms for spermatodose size, and number of competing males were not significant ($P > 0.25$), whereas such a term was significant for relative mating order (Table 5). Both a larger spermatodose size and being later in the mating order increased the chance of siring offspring (Fig. 3A, B, Table 5). The effect of relative mating order followed a quadratic curve, further penalizing males early in the mating order. A higher number of competing males reduced the chances of siring offspring (Fig. 3C, Table 5).

When assigning paternity to males, we provided MasterBayes with phenotypic information (mating order and spermatodose size). As MasterBayes simultaneously estimates the pedigree and the population-level parameters, there should be no bias from the use of this approach on our subsequent analysis to examine the effects of mating order and spermatodose size on proportion of offspring sired. To demonstrate this, we

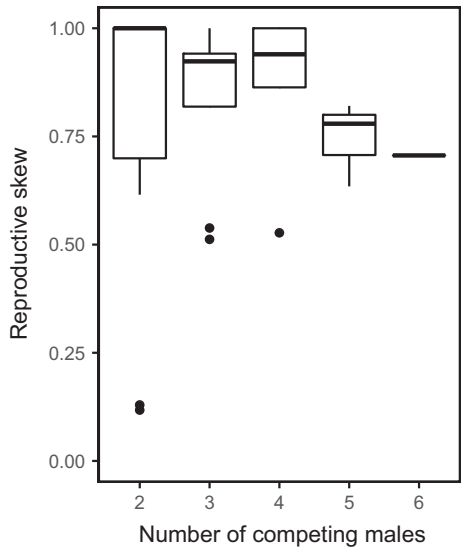

Fig. 2 Paternity skew for different numbers of competing males. A value of 1 indicates all offspring of a female are sired by one male, whereas a value of 0 indicates all males sire the same number of a female's offspring.

Table 5 Parameter estimates from the best-fitting quasi-Poisson GLM, showing the effects of relative spermatodose order within the spermatheca, number of competing males and spermatodose diameter on paternity

Coefficients	Estimate	*t* Value	*P*-value
Relative order	3.65	3.09	0.0026
(Relative order)^2	−2.70	−2.52	0.0132
Number of competing males	−0.39	−3.51	0.0007
Spermatodose diameter	2.75	4.01	0.0001

repeated our analysis when paternity was estimated without any phenotypic information (i.e. assigning paternity using only genotypes). This approach produced very similar results to those described above (Table S1, Supporting information).

Overall 44 of 105 males (spermatodoses) produced 0 offspring. The proportion of males that sired no offspring was higher in earlier mating males (proportion of males siring no offspring when mating males last: 0.294, intermediate: 0.395 and first: 0.576); however, these differences were nonsignificant (logistic regression, $P > 0.05$). Interestingly, we found that when the last male to mate sired no offspring, the male mating second-to-last sired most of the female's offspring (mean proportion of offspring sired = 0.63).

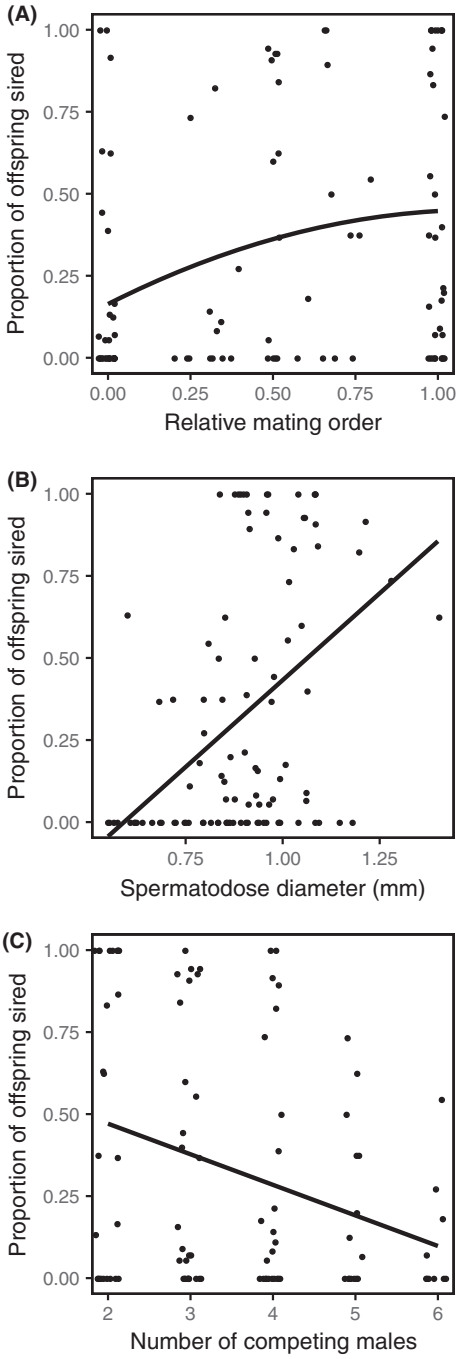

Fig. 3 The relationships between the proportion of offspring sired by a given male and (A) the position of the male's spermatodose within the spermatheca (0 = furthest from the single exit/entrance, 1 = closest to the entrance/exit); (B). the diameter (in mm) of the male's spermatodose and (C) the number of competing males (see also Table 5). Note points were jittered along the *x*-axis to aid visualization of overlapping points.

Discussion

Here, we have examined the influence of spermatodose size and placement on paternity in field-collected samples of *P. griseoaptera*. Paternity share was highly skewed with typically only one or two males siring the majority of a female's offspring. Both the size and

relative order of the spermatodoses within the spermatheca had a significant effect on paternity, with a bias against smaller spermatodoses and those further from the single entrance/exit of the spermatheca. As expected, a higher number of competing males also reduced the chances of siring offspring for each male. While previous studies of orthopteran insects have used microsatellite analysis to estimate the degree of polyandry and paternity skew in field-mated females (Hockham *et al.* 2004; Bretman & Tregenza 2005; Simmons *et al.* 2007; Simmons & Beveridge 2010; Oneal & Knowles 2015; Turnell & Shaw 2015a,b), none of these have used the relative position of sperm within the female's reproductive tract to predict the pattern of sperm use. Even if laboratory-based studies and other taxa are included, the number of previous studies that have been able to relate directly the relative position of sperm within the female's reproductive tract to sperm use by the female is very limited (Droge-Young *et al.* 2016). Manier *et al.* (2010, 2013a,b) and Droge-Young *et al.* (2016), for example, used transgenic lines with fluorescent-tagged sperm heads to resolve mechanisms of competitive fertilization success in *Drosophila* spp and *Tribolium casteneum*, respectively, in a laboratory setting.

A further novel aspect of the present study was that, in the absence of field observations, we were able to determine for each female the extent of repeated as opposed to multiple mating. Our results indicated that there was a very low frequency of repeated mating with the same male (only six of 36 females contained two spermatodoses of the same genotype, and no females contained >2 spermatodoses of the same genotype). Furthermore, there was only one case of a female that appeared to have mated twice with the same male in two successive matings (note that this may be considered a conservative estimate, because it is possible that two males could share the same genotype). This low remating rate could be a result of the 5-day-long sexual refractory period in the female (Supporting information) as a male that mates with a female is likely to have moved on by the time the female is ready to mate again. The low level of repeated mating with the same male could also reflect female choice (Ivy *et al.* 2005; Weddle *et al.* 2013). Laboratory mate choice trials in Gryllid crickets, such as *Gryllodes sigillatus*, have demonstrated that females actively avoid copulating with previous mates, presumably to obtain any benefits from mating with different males (see Ivy *et al.* 2005; Weddle *et al.* 2013).

The relationship between spermatodose position within the spermatheca and paternity in the present study was best explained by a quadratic curve; while spermatodoses furthest away from the opening of the spermatheca were generally less successful in achieving paternity, there were diminishing returns of being positioned closer to the spermathecal opening. This pattern is not entirely consistent with the hypothesis that spermatodoses allow the male to block the exit of rival sperm already present within the spermatheca (Simmons & Siva-Jothy 1998), which would predict paternity to be very strongly skewed in favour of the last male to mate. Sperm from all spermatodoses, even those at the distal end of the spermatheca (i.e. from male that mated first), achieved some paternity.

Because sperm in storage were examined, some mechanisms of sperm precedence can be ruled out, such as the removal or ejection of sperm from previous males (Simmons & Siva-Jothy 1998; Simmons 2001). It is, however, possible that females may have used up a greater proportion of sperm from earlier matings by the time they were collected. Furthermore, in common with virtually all other studies of sperm precedence, the possibility that postmeiotic sperm ageing might have contributed to the patterns of sperm use observed cannot be ruled out (Pizzari *et al.* 2007). The likely time that sperm were in storage in proportion to the female's lifespan was relatively short, however. The median number of matings for females in the present study was 3. Given that females have a sexual refractory period of 5 days (Supporting information), that the majority of mating in this species occurs in August and that females were collected in early September, a reasonable estimate of the time that sperm had been in storage in the spermatheca would be in the region of 10–20 days. In contrast, the adult lifespan of the female is likely to be 3–4 months or more; females can frequently survive and continue to lay eggs into October and November, or even later (Hartley & Warne 1972; Benton 2012).

The only data available on sperm precedence in another tettigoniid species that produces spermatodoses examined patterns of sperm precedence of female *Decticus verrucivorus* (which is in the same subfamily as *P. griseoaptera*) that had mated with two different males in a laboratory setting (Wedell 1991). Results were consistent with a 'fair raffle' (Parker 1990) and, unlike in the present study, no bias against the use of sperm from the first male to mate was reported. It is possible that depletion or ageing of sperm from the first mating could have been more pronounced in our study in comparison with that of Wedell (1991), which could have contributed to the observed fertilization bias against earlier spermatodoses. Future work comparing paternity patterns in both the field and laboratory will help to resolve these issues.

Unexpectedly, approximately one-third of the spermatodoses closest to the exit/entrance of the spermatheca sired no offspring. In many insects, mating failures are known to occur (Greenway & Shuker 2015). Such

failures are often interpreted as resulting from a failure to transfer sperm to the female's sperm storage organs, which was clearly not the case here. When dissecting spermatodoses, it was apparent that some still appeared to be full of a large ball of tightly coiled spermatodesmata, while others appeared to be almost empty (Vahed 2003). It is possible that spermatodoses do not begin to release their content immediately, but that there is a delay. Even if discharge from the spermatodoses does begin soon after their transfer, those from the females' most recent mates would have had less time to discharge their content into the spermatheca, perhaps accounting for the relatively high proportion of offspring sired by sperm from spermatodoses in the second-to-last mating position in these families. The mechanism by which sperm are released from spermatodoses and the rate at which they are discharged is currently unknown (Vahed 2003). A further possible reason why sperm from spermatodoses closest to the exit of the spermatheca did not always achieve highest paternity relates to the position of the elongated spermatodose tube (through which sperm exit the spermatodose). Vahed (2003) observed that in *P. griseoaptera*, in 50% of cases, the spermatodose tube of the spermatodose nearest to the spermathecal exit was oriented away from the exit rather than towards it.

In some cricket species, there is compelling evidence that the female can bias the use of sperm from selected males by controlling not only the duration of attachment of an externally attached spermatophore, but also the uptake of sperm to the spermatheca (Vahed 2015). Whether or not the female can influence the discharge of sperm from spermatodoses as a further mechanism of cryptic female choice deserves further investigation. There is also evidence that females might be able to exert control over the differential storage and use of sperm from their mates by digesting stored sperm. In some bushcrickets, for example, spermolytic activity has been found within the lumen of the duct of the spermatheca (Viscuso *et al.* 1996; Brundo *et al.* 2011). It has been proposed that the walls of the spermatodoses may function to protect the male's sperm from such spermolytic activity within the spermatheca (Vahed 2003); that is, spermatodoses may be the result of intersexual conflict over the fate of stored sperm, and sperm in older spermatodoses may be more degraded as well as further away from the spermathecal opening.

We found that sperm from larger spermatodoses had a greater chance of siring offspring. This is consistent with other sperm competition studies of various taxa, which have demonstrated that when a female has mated with two different males, the relative number of sperm from each male predicts the paternity of her offspring (Martin *et al.* 1974; Simmons 1987; Parker *et al.* 1990; Wedell 1991; Gage & Morrow 2003; Bretman *et al.* 2009). Spermatodose size is highly likely to reflect sperm number: when full, the sperm occur in a tightly coiled ball which takes up most of the spherical body of the spermatodose (Vahed 2003). The transfer of larger volumes of ejaculate does not only benefit the male by increasing his representation in the female's sperm stores. Evidence suggests that in many insects, including bushcrickets, substances in the ejaculate are also transferred that delay the female from remating in a dose-dependent manner (Gillott 2003). This effect might also be triggered by an increase in the physical 'fullness' of the spermatheca. In *P. griseoaptera*, Jarčuška & Kaňuch (2014) found that the mean size of spermatodoses within the spermatheca predicts the number of spermatodoses received over the female's lifetime, suggesting that females that had received a larger ejaculate subsequently mated with fewer males. We were unable to confirm this relationship using our data set, although it should be noted that the sample size of females was smaller than in Jarčuška & Kaňuch's (2014) study. The benefit to a male of delaying or deterring his mate from remating was demonstrated in the present study: we found that the proportion of offspring sired by each male declined with the number of competing males. Simmons & Beveridge (2010) found a similar pattern in the field cricket *Teleogryllus oceanicus* that had mated in the field.

It is possible that the influence of spermatodose order on paternity varies with differences in polyandry. In *P. griseoptera*, we found that females contained up to 6 spermatodoses (median = 3); however, the number of spermatodoses per female (i.e. the degree of polyandry) is considerably greater than this in some bushcrickets (Vahed 2006). In *Platycleis affinis*, for example, females contained up to 23 spermatodoses, while in *Anonconotus* spp, females contain up to 44 (Vahed 2006). Examining the influence of spermatodose order on paternity in such highly polyandrous species would be challenging but potentially useful. In addition, the lifetime degree of polyandry is known to vary between populations [e.g. clinal variation in remating rate is seen in *Drosophila pseudoobscura* (Price *et al.* 2008) and *Metrioptera roeselii* (Kaňuch *et al.* 2013)]. The techniques used here could be used to compare how mating order affects sperm precedence between different populations, which could provide a novel means of testing models of ejaculate allocation (e.g. Parker 1990, 1998).

Using a species in which sperm from different matings occur within discreet aggregations (spermatodoses), we were able to examine the effects of the order of sperm deposition from different males within the female's sperm storage organ and of ejaculate size, on male fertilization success in females that had mated

under natural field conditions. The approach used here is likely to be generalizable to other taxa in which sperm form discrete aggregations, but perhaps also to taxa for which the stratification of sperm due to mating order may be more cryptic. Future work to examine the influence of sperm aggregation on paternity is needed, in particular from species in which sperm aggregations are less discreet (e.g. see Mann 1984; Higginson & Pitnick 2011; Fisher *et al.* 2014).

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Data accessibility

All data are available in Dryad: http://datadryad.org/review?doi=doi:10.5061/dryad.6t3dn.

D.J.P., M.G.R and K.V. conceived of the study and designed the research. K.V. conducted fieldwork and dissected *Pholidoptera* females. J.Z. and D.J.P. performed the laboratory work. D.J.P. and J.Z. analysed the data, with input from M.G.R and K.V. D.J.P., M.G.R and K.V. wrote the manuscript with input from J.Z.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Additional methods used to extract DNA from spermatodose samples, and to estimate male and female refractory periods. Values for male and female refractory periods are also reported here.

Table S1 Parameter estimates from the best-fitting quasi-Poisson GLM, showing the effects of relative spermatodose order within the spermatheca, number of competing males, and spermatodose diameter on paternity when paternity was assigned independently of any phenotypic information.